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**Development of a three tier *in vitro* system, using Caco-2 cells, to assess the effects of lactate on iron uptake and transport from rye bread following *in vitro* digestion.**

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Running title: Effects of lactate on iron absorption in 3-tier *in vitro* digestion/Caco-2 model

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## ABSTRACT

We have developed a 3-tier Caco-2 cell system to simultaneously assess iron dialyzability, uptake and transport across the Caco-2 monolayer from an *in vitro* digested food matrix. The effect of lactate (0–200 mmol L<sup>-1</sup>) on iron absorption from rye bread subjected to simulated peptic (pH 5.5) and pancreatic digestion (pH 6.5) was investigated to model absorption pre- and post the sphincter of Oddi. Lactate increased dialyzability (11.8%,  $P < 0.05$ ) in peptic digests whereas it reduced it in pancreatic digests (4.9%,  $P < 0.001$ ). Iron uptake from the peptic digests was in the region 39–76 pmol mg protein<sup>-1</sup> whereas it decreased from 281 to 51 pmol mg protein<sup>-1</sup> in pancreatic digests. Iron transport was calculated for the peptic digests from <sup>14</sup>C-polyethylene glycol movement, and only at 200 mmol L<sup>-1</sup> lactate was there any detectable transcellular transport (180 pmol mg protein<sup>-1</sup>,  $P < 0.05$ ). Iron absorption was positively correlated to dialyzable iron for both digests ( $R^2 = 0.48$  and  $R^2 = 0.41$ , respectively,  $P < 0.01$ ), and the effect of lactate was therefore associated mainly with iron bioaccessibility. The 3-tier system showed the potential to obtain detailed insight into each step involved in iron transport across the monolayer from a food mixture.

Keywords: Iron bioavailability, Caco-2, *in vitro* digestion, lactate, phytate

## INTRODUCTION

Predictive methods for assessing iron bioavailability from complex mixtures are useful tools for testing the modulating effect of dietary factors. A number of steps influence how much iron enters the body: (a) iron solubility in the food digest, (b) the concentration of iron that is in contact with the epithelial layer, its valency, and whether it is bound to small organic ions that inhibit or enhance release from the food matrix, (c) the mucosal “setting”, which is dependent on the iron status of the body and regulates the efficiency of enterocyte uptake and exit of iron from the cell into the systemic circulation, and (d) modulators of paracellular transfer.<sup>1</sup>

The Caco-2 cell model has been used to assess iron absorption from complex foods subjected to simulated digestion prior to adding a soluble fraction to the cell monolayer on a semi-permeable membrane, which permits the measurement of both uptake and transport across the membrane.<sup>2,3</sup> Glahn *et al.*<sup>4</sup> measure iron uptake into the cells by inserting a dialysis membrane above a cell monolayer grown on the base of the cell culture well, simulating the mucous layer that protects the cells from the digestive enzymes. One disadvantage of this method is that it cannot be used to predict exit from the cell into the plasma. Also, the fact that the efflux pathway is blocked may alter the uptake rate of iron into the cell. Dietary iron absorption is often correlated with the size of the iron stores, indicating that the stores regulator, which modulates intestinal iron absorption in response to the level of body iron stores,<sup>5</sup> is an important factor in predicting iron bioavailability. Extending the Caco-2 cell model to a 3-tier system, including both a dialysis membrane and the epithelial monolayer grown on a semi-permeable membrane, has the potential to simulate a range of iron status scenarios in

the model, for example, by adding transferrin with various degrees of iron saturation<sup>6</sup> in the basolateral chamber or the recently identified regulator of iron absorption, hepcidin.<sup>7</sup>

Predicting iron bioavailability from a food matrix is difficult, as it depends on several factors associated with food composition and structure. In particular, phytate and polyphenols are significant inhibitors of iron bioavailability.<sup>8,9,10</sup> Fermentation of vegetables and cereals is known to increase nonheme iron absorption, possibly due to the production of small organic acids.<sup>11,12,13</sup> Suggested mechanisms for this effect include the lowering of pH and increased buffering capacity, which may hinder or delay the formation of less soluble compounds,<sup>11</sup> activation of phytases,<sup>10</sup> chelation of iron with organic acids, hindering both complex binding to phytate and precipitation in the gut,<sup>12</sup> and finally the delayed gastric emptying rate caused by the organic acids, which may increase exposure time of iron to the gut.<sup>14,15</sup>

The aim of the current study was to assess the feasibility of using a 3-tier Caco-2 cell system using *in vitro* digestion in the tier above a dialysis membrane and measuring iron uptake and transport across the monolayer. We assessed the suitability of this technique by investigating the effect of different concentrations of lactate, on iron absorption from a phytate rich meal using a food matrix of rye bread. Levels equivalent to those achieved during fermentation of sourdough and sauerkraut<sup>16,17</sup> were selected so as to assess whether the increased iron absorption from fermented vegetables and cereals seen in humans related to the presence of organic acids. We chose to use bread as a typical model food matrix to test in the system as this would be closer to the envisaged end use compared to a simple buffer; but then to add different concentrations of lactate to the bread, rather than ferment the bread, so that the phytate content

1 remained unchanged and to test only one variable in the system. It is known that phytate  
2 influences iron uptake into Caco-2 cells independently of any effect on dialyzability.<sup>18</sup>  
3 As iron is primarily absorbed in the duodenum and the most proximal part of jejunum<sup>19</sup>  
4 the study was set up to measure iron absorption from a peptic (pH 5.5) and a pancreatic  
5 (pH 6.5) *in vitro* digest, corresponding to the digestion conditions in the lumen of the  
6 proximal duodenum and jejunum respectively.<sup>20</sup>

## **MATERIALS AND METHODS**

### **Chemicals**

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or BDH Laboratory Supplies (Dorset, UK).

### **Cell Culture**

Caco-2 cells were obtained from the European Collection of Cell Cultures (Wiltshire, UK). Cells at passage 47 were seeded at a density of  $10^4$  cells  $\text{cm}^{-2}$  onto 6-well Transwell polycarbonate membranes with a pore size of  $0.4 \mu\text{m}$  and an area of  $4.7 \text{ cm}^2$  (Costar Corp., Cambridge, MA) and grown at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  in standard growth media: Dulbecco's Modified Eagle Medium (Sigma D-6429) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine,  $100 \text{ U mL}^{-1}$  penicillin and  $100 \mu\text{g mL}^{-1}$  streptomycin. Culture media was changed every 2–3 days. Iron absorption studies were conducted 19 days post seeding. These conditions were based on preliminary studies showing that at this time maximal iron transport had been reached and high transepithelial electrical resistance (TEER) readings were consistently measured.

### **Preparation of the 3-tier system**

The membranes were removed from 12 mm transwell inserts (Costar), which instead were fitted with a dialysis membrane (cut-off 12-14 kDa, Spectra/Por 2, Spectrum Laboratories, Inc., Rancho Dominguez, CA), fixed by a silicone ring (14 mm internal diameter, purchased locally). The inserts were sterilized in 70% ethanol and washed in sterile water before use. The inserts were placed above the 24 mm transwell membranes (6-well plates) by use of insert rings (23 mm internal diameter, 36 mm external diameter, 0.5 mm high, polytetrafluoroethylene), thereby creating the 3-tier system presented in Fig 1.

## ***In vitro* digestion**

Samples of 50 mg mL<sup>-1</sup> rye bread solutions (2.85 µmol inositol phosphates (IP)<sub>3-6</sub> mg dry matter<sup>-1</sup>, containing 6% IP<sub>3</sub>, 7% IP<sub>4</sub>, 14% IP<sub>5</sub>, and 73% IP<sub>6</sub>) were prepared by mixing 200 mg freeze-dried, homogenized rye bread in 120 mmol L<sup>-1</sup> NaCl and adding L-lactic acid to a final concentration of 0–200 mmol L<sup>-1</sup>. Samples were spiked with 900 kBq <sup>55</sup>FeCl<sub>3</sub> (Specific activity 717 MBq mg<sup>-1</sup>, PerkinElmer Life Sciences, Inc., Boston, MA) and equilibrated for 10 min. The pH was adjusted to 2.0 (with 2.0 mol L<sup>-1</sup> HCl) and the volume was made up to 4.0 mL prior to the addition of 0.2 mL pepsin solution (40 mg mL<sup>-1</sup> porcine pepsin, 600–1,800 units mg protein<sup>-1</sup>, in 0.1 mol L<sup>-1</sup> HCl), and the samples were incubated 1 h at 37°C (200 rpm) in an incubator (Innova 4400, Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ). After the pepsin digestion the samples were divided into two. In the first 2.0 mL, the pH was raised to 5.5 by dropwise addition of 5.0 mol L<sup>-1</sup> NaOH, and the volume adjusted to 2.9 mL with 120 mol L<sup>-1</sup> NaCl. These digests were stored at -20°C until iron absorption experiments. To the final 2.0 mL, 0.5 mL pancreatin-bile solution (0.4 mg mL<sup>-1</sup> porcine pancreatin, 4 × USP specifications, and 2.4 mg mL<sup>-1</sup> porcine bile extract in 120 mmol L<sup>-1</sup> NaCl) was added, and the pH raised to 6.5 by dropwise addition of saturated NaHCO<sub>3</sub>. The volume was adjusted to 2.9 mL with 120 mmol L<sup>-1</sup> NaCl. These digests were transferred directly to the 3-tier system for iron absorption experiments.

## **Iron uptake and transport by Caco-2 cells**

Immediately before the pancreatic digestion period, medium was removed from the cells and they were washed with 2 × 2 mL Hank's Balanced Salt Solution (HBSS), pH 6.5. Then 1.5 mL and 1.0 mL HBSS, pH 6.5 were added to the cell and basolateral compartments, respectively. The transwell inserts with dialysis membranes were placed above the cell monolayers and 0.5 mL of the pancreatic digests was placed in these



1 inserts. The plates and residuals of the intestinal digests (control for determination of  
2 total soluble iron in the food digests) were incubated for 2 h at 37°C (65 rpm) in an  
3 Innova 4400 incubator, then the digests, the dialysates and the basolateral solutions  
4 were collected for determination of  $^{55}\text{Fe}$ . Non-absorbed iron was removed from the cell  
5 layers according to Glahn *et al.*<sup>21</sup> Briefly, the cell layers were washed in 2 × 2 mL  
6 “rinse” solution (140 mmol L<sup>-1</sup> NaCl, 5 mmol L<sup>-1</sup> KCl, and 10 mmol L<sup>-1</sup> piperazine-N-  
7 N’-bis(2-ethanesulfonic acid) (PIPES), incubated 10 min with 2 mL “removal” solution  
8 (rinse solution with an additional 1 mmol L<sup>-1</sup> bathophenanthroline disulfonic acid and 5  
9 mmol L<sup>-1</sup> sodium hydrosulfite) and washed with 2 × 2 mL rinse solution. Finally, the  
10 cells were harvested with 1.5 mL, 200 mmol L<sup>-1</sup> NaOH for determination of  $^{55}\text{Fe}$  and  
11 protein. Absorption studies with the peptic digests were performed in a similar manner.

## 12 **Assessment of paracellular transport**

13 After the pepsin digestion and the subsequent division of samples in two, 65 kBq  $^{14}\text{C}$ -  
14 polyethylene glycol (PEG) 4000 (Specific activity 581 MBq g<sup>-1</sup>, Amersham Pharmacia  
15 Biotech, Buckinghamshire, UK) was added to each tube to assess paracellular transport  
16 across the monolayer.

## 17 **Analyses**

18 Activities of  $^{55}\text{Fe}$  and  $^{14}\text{C}$ -PEG was determined by dual isotope liquid scintillation  
19 counting of samples in aqueous solution with 90% Quicksafe A (Zinsser Analytic  
20 GmbH, Frankfurt, Germany) in a Tri-Carb 2700TR (Packard Instruments, Meriden,  
21 CT). The scintillation counting was performed with corrections for quenching and  
22 window settings 0–10 and 12–156 keV for  $^{55}\text{Fe}$  and  $^{14}\text{C}$ -PEG, respectively. Cellular  
23 protein was determined using the Bicinchoninic Acid Protein Assay Kit (Pierce  
24 Biotechnology, Rockford, IL). Total soluble iron was determined as described by  
25 Kapsokefalou and Miller.<sup>22</sup> Briefly, reducing protein precipitant solution (200 µL) was

added to 400  $\mu$ L aliquots of each control digest sample. Samples were held overnight at room temperature. Subsequently they were centrifuged at  $2575 \times g$  for 10 min. Aliquots of the supernatants (100  $\mu$ L in duplicate) were transferred to a 96-well plate and 200  $\mu$ L N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer pH 9.9 and 25  $\mu$ L ferrozine solution were added to each well. Absorbance (570 nm) was measured after 1 h incubation, and sample iron concentrations were calculated from data generated from standards. The content of phytate in the rye bread was measured as individual IP<sub>3-6</sub> by high-performance ion chromatography.<sup>23</sup> The quantities of total dialyzed iron, iron uptake and transport by the cells were calculated from the specific activity of <sup>55</sup>Fe (amount of radioactivity per mol of soluble iron) in the digest control samples (stationary system in which no iron has been removed or entered) and the activity of <sup>55</sup>Fe measured in the digests, dialysates, cells and transport solutions of the 3-tier system. The amount of iron in the dialysate is given as nanomoles per litre and converted to percentage of the initial concentration in the digest. Iron uptake and transport are given as picomoles per milligram of cell protein. The percentage of iron transported paracellularly from the pepsin digests was then calculated from the proportion of <sup>14</sup>C-PEG in the dialysate which crossed the epithelial layer, with the assumption 'tight junctions' are equally permeable to iron and PEG

### **Statistical analysis**

For each food sample four replicates were made with independent digests and plates for each replicate and data given as mean  $\pm$  SEM,  $n = 4$ . The effects of lactate or iron content on iron movement between compartments were assessed by ANOVA with Bonferroni post test and linear regression analysis of all data points using Prism 3.0 (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, USA). Means were considered significantly different for  $P < 0.05$ .

## RESULTS

### Effects of lactate on soluble and dialyzable iron

Following the pepsin digestion the concentration of soluble iron in the digest was  $8.2 \pm 1.2 \mu\text{mol L}^{-1}$  while the equivalent concentration following the pancreatin digestion was significantly higher,  $11.2 \pm 1.1 \mu\text{mol L}^{-1}$  ( $P < 0.001$ ), with no effect of lactate on these parameters. The concentrations of iron in the apical compartment below the dialysis membrane (dialysate) after 2 h, and the percentage of the total amount of iron dialyzed (sum of iron in the apical, cell, and basolateral compartment) relative to the total iron in the 3-tier system (sum of iron in the digest, apical, cell, and basolateral compartment) are shown in Fig 2. The amount of iron dialyzed from the peptic digests increased significantly from 11.9% to 23.7% ( $P < 0.05$ ) with increase in lactate from 10 to 200  $\text{mmol L}^{-1}$ , showing significant correlation with lactate concentration ( $R^2 = 0.49$ ,  $P < 0.005$ ). In contrast, following the pancreatin digestion, lactate (200  $\text{mmol L}^{-1}$ ) caused a significant reduction in the percentage iron dialyzed from 6.9% to 2.0% ( $P < 0.001$ ) with an inverse relationship between dialyzable iron and lactate concentration ( $R^2 = 0.51$ ,  $P < 0.001$ ).

### Effects of lactate on iron uptake

Iron uptake into the cells over the 2 h in which the pepsin digestion was carried out in the upper chamber was in the region of 39 to 76  $\text{pmol mg protein}^{-1}$  in the presence of 0 to 200  $\text{mmol L}^{-1}$  lactate in the digests (1.2 to 2.8% of the total soluble iron) with no effect of increasing lactate concentration (Fig 3). During the pancreatin digestion stage, iron uptake decreased from 228 to 81  $\text{pmol mg protein}^{-1}$  with an increase in lactate concentration from 0 to 10  $\text{mmol L}^{-1}$ , a decrease from 4.9% to 1.7% of the total soluble iron in the system ( $P < 0.001$ ). Further reductions in uptake with increasing lactate

concentration occurred, so that at 200 mmol L<sup>-1</sup> only 0.2% of the iron in the system was taken up by the cells (Fig 3).

### Effects of lactate on iron transport

During the pepsin digestion phase the amount of iron in the basolateral compartment increased with increasing lactate concentrations such that twice as much iron crossed the epithelial layer in the presence of 200 mmol L<sup>-1</sup> lactate as when no lactate was present. The amount of iron reaching the basolateral side was proportional to dialyzable iron concentrations ( $R^2 = 0.87$ ,  $P < 0.001$ ) but much of this transported iron could be accounted for by paracellular transport as estimated using the <sup>14</sup>C-PEG levels in the dialysate. Overall  $15.3 \pm 1.8\%$  ( $n = 20$ ) of the <sup>14</sup>C-PEG crossed the dialysis membrane during the peptic incubation period. This compares to  $12.0 \pm 1.2\%$  ( $n = 20$ ) of the labelled iron added to the food matrix. From the relative concentrations of <sup>55</sup>Fe and <sup>14</sup>C-PEG in the dialysate we calculated the amount of transfer across the Caco-2 cell layer, which could be assigned as paracellular transport and by difference the amount that was transported transcellularly. Only at 200 mmol L<sup>-1</sup> lactate ( $0.9 \mu\text{mol iron L}^{-1}$  dialysate) was there any significant transcellular transport ( $180 \text{ pmol mg protein}^{-1}$ ,  $P < 0.05$ ). The total iron uptake (total iron crossing the apical membrane) can be calculated by adding this transcellular transport to the amount of iron uptake into the cell at 2 h. This calculation would then suggest that during the peptic digestion there is a positive linear relationship between dialyzable iron and total iron uptake ( $R^2 = 0.48$ , respectively,  $P < 0.01$ ), as shown in Fig 4.

Following the 2 h pancreatin digestion phase only  $53 \text{ pmol iron mg protein}^{-1}$  was transported to the basolateral side with no effect of lactate. It was not possible to calculate the relative impacts of paracellular and transcellular iron transport as, following the addition of bile acids and pancreatin, only a negligible amount of <sup>14</sup>C-

PEG was able to cross the dialysis membrane,  $0.67 \pm 0.14\%$  ( $n = 20$ ), although  $2.6 \pm 0.2\%$  ( $n = 20$ ) of iron was dialysed. Iron absorption in relation to dialyzable iron is shown for the pancreatic digests in Fig 4 as well, but these absorption values only includes uptake rates, as the transcellular transport was not determined. The transport was however minor in these digests and not influenced by lactate concentration, so that overall there was a positive linear relationship between dialyzable iron and total iron uptake during the pancreatin digestion step as well ( $R^2 = 0.41$ ,  $P < 0.01$ ). During the pancreatic digestion, at the higher pH (6.5 v. 5.5) a higher fraction of the dialyzed iron was taken up into the cells than during the pepsin digestion step (Fig 4).

## DISCUSSION

The use of the 3-tier Caco-2 cell system to allow measurement of soluble and dialyzable iron together with iron uptake and transport, all in one experiment provides a valuable and novel approach to the assessment of bioavailability, and offers a further refinement of the model developed by Glahn *et al.*<sup>4</sup> A second novel aspect of this study is the experimental determination of what happens to iron when food leaves the stomach, before it is mixed with pancreatic secretions at the sphincter of Oddi. The upper part of the duodenum is recognized to be the area of maximal expression of brush-border associated divalent metal transporter (DMT1) activity, especially following iron deprivation.<sup>24</sup> The residence time of material in the duodenum is variable but comparatively short following a meal, but as the free iron is probably still soluble due to the low pH, and as food may empty from the stomach over a period of several hours, the cells covering the duodenal wall will be exposed to pepsin digested food for a prolonged period, and a significant part of the dietary iron could be taken up here. For molecules to cross the mucous layer they must be present in low molecular weight hydrophilic forms. In the 3-tier system this step is assessed by analyzing the proportion of the soluble iron present in the food matrix that crosses the dialysis membrane. This dialyzable iron will depend on the pH of the food matrix,<sup>25</sup> phytate content and the presence of small organic acids such as citric acid and ascorbic acid,<sup>26</sup> and probably also the lactate content, as investigated in our study.

The addition of lactate (0–200 mmol L<sup>-1</sup>) had no effect on the soluble iron content of the food matrix in either the peptic (pH 5.5) or pancreatic (pH 6.5) digests. However, the presence of increasing concentrations of lactate increased the amount of iron dialyzed from peptic digests, leading to concentrations of iron within the dialysate in the region

of 0.9  $\mu\text{mol L}^{-1}$ . In contrast, following adjustment to pH 6.5 and digestion with pancreatin-bile, dialyzable iron concentration was reduced to 0.1  $\mu\text{mol L}^{-1}$ , and in this situation lactate further reduced the concentrations of dialyzable iron, presumably by increasing the amount of iron complexed into particles larger than the molecular weight cut-off of the dialysis membrane while still being effectively soluble. The improved dialysability of iron, at pH 5.5, on addition of lactate may reflect competition between the phytate naturally present in the rye bread and the lactate as previously suggested by Salovaara *et al.*<sup>27</sup> The concentration of iron in the dialysates in these experiments is therefore more than 2 orders of magnitude less than those routinely used to study transport kinetics.<sup>28,29</sup> Therefore, saturation of key transporter activity is unlikely, and the linear relationship between the concentration in the dialysate and uptake into the cell reported here would be predicted, particularly as phytate concentrations were standardised. Most food matrices used previously in Caco-2 experiments to assess nonheme iron absorption have a total nonheme iron content in the region of 8–33  $\mu\text{mol L}^{-1}$  and a dialysate concentration of 0.5–4  $\mu\text{mol L}^{-1}$ ,<sup>2,4,30</sup> a more physiologically meaningful range for the luminal content in the proximal small intestine and similar to those used in the current study. A linear relationship between dialyzed iron and apparent absorption is seen for both peptic and pancreatic digests (Fig 4). This also explains why low concentrations of lactate (< 50 mmol  $\text{L}^{-1}$ ) decreased iron absorption, as less iron was dialyzed from these samples (% iron dialyzed, Fig 2) than from samples without lactate. The inhibitory effect of lactate on iron uptake from the pancreatic digests corresponds well with the inhibitory effect of lactate on iron uptake in Caco-2 cells described previously<sup>31,32</sup> using solutions of lactate and iron at pH 6.8 and 7, respectively, and our results would suggest that the inhibitory effect of lactate may be associated mainly with a decrease in iron bioaccessibility, reflected in our study as

1 dialyzability, rather than an effect on solubility and therefore a lower availability for  
2 absorption.

3 In the case of the peptic digest (pH 5.5) the amount of iron uptake into the cell does not  
4 correlate with either the concentration in the dialysate or the basolateral media. This  
5 might suggest that intracellular iron is not always a good indicator of bioavailability as  
6 has been suggested previously.<sup>18</sup> The only significant positive effect of lactate on iron  
7 absorption from the peptic digest was seen at the highest lactate concentration (200  
8 mmol L<sup>-1</sup>). This concentration is in the upper range of what is seen in lactic acid  
9 fermented foods that are high in lactate,<sup>33,16,17</sup> and taking into consideration the 2–3 fold  
10 dilution of the food by gastric and intestinal secretions our data suggests that it is  
11 unlikely that lactate will increase iron absorption from a meal. It is perhaps surprising  
12 that the fractional uptake of dialyzable iron is higher following the pancreatic digestion,  
13 where pH was higher, than after the peptic digestion (Fig 4). As more iron is soluble at  
14 the lower pH and as iron enters the cells across the apical membrane via DMT1, which  
15 is a proton-coupled process and depends on the cell membrane potential,<sup>34</sup> it would be  
16 predicted that iron absorption from the peptic digests, with the lower pH, would be  
17 greater. The fact that it does not suggests that either the lower pH of the peptic digests  
18 makes the Caco-2 cells less efficient, or perhaps more likely the pancreatic digestion  
19 removes dialyzable, but inhibitory factors, from the food matrix, such as iron-peptide  
20 complexes, and thereby increase iron uptake.

21  
22 This initial study using the 3-tier Caco-2 cell model demonstrated that this system is  
23 promising in studying iron uptake and transport from complex food matrices  
24 undergoing digestion *in vitro*. The presence of lactate affected the amount of iron  
25 dialyzed more than the transport, and thus highlights the importance of the dialysis



1 membrane in the system. It is probable that limiting export of iron from the cells in a  
2 monolayer grown on the plastic base of the well would inhibit uptake, as a response to  
3 increased intracellular iron,<sup>35</sup> and therefore also to the relation between dialyzed iron  
4 and iron uptake. We therefore included the monolayer step in our model, but our PEG  
5 data suggest the continuity of the ‘epithelium’ may have been compromised. To allow  
6 for this in future, and to maximise the value of the system, kinetic transport studies of a  
7 marker of passive transport, such as mannitol, should be made during the incubation  
8 period so that permeability of the monolayer can be accounted for in the transport  
9 measurements. The main purpose of the 3-tier model is to be able to measure iron  
10 absorption from foods in a system in which the mucosal “setting” of the epithelial layer,  
11 reflecting the iron status of individuals, is the main determinate of iron transport,  
12 independent of the form of iron in the food. It still remains to be investigated how  
13 serosal factors affect the transepithelial transport in the 3-tier system, i.e. the plasma  
14 protein transferrin as an acceptor of iron<sup>36</sup> or hepcidin as a regulator of iron transport.

15  
16 In conclusion, the 3-tier system described in this paper has the potential to obtain  
17 detailed insight into each step involved in mineral transport across the intestinal  
18 epithelium from food mixtures, thus providing a useful tool for designing foods with  
19 maximal bioavailability.

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## FIGURE LEGENDS

**Figure 1** Diagram of the experimental model. The model includes *in vitro* digestion with either pepsin or pepsin and pancreatin-bile (directly in the digest compartment) followed by uptake and transport studies in the 3-tier Caco-2 cell system. The system consists of 4 compartments, i.e. the digest (the compartment above the dialysis membrane), dialyzable digest (apical compartment below the dialysis membrane), cell monolayer and basolateral compartment.  $^{55}\text{Fe}$  is used as a marker of iron uptake and transport and  $^{14}\text{C}$ -PEG as a non-absorbable marker of monolayer tightness.

**Figure 2** Iron dialysate concentration following a 2 h incubation of a rye bread digest in the presence of increasing amounts of lactate from 0–200 mmol L<sup>-1</sup> initial concentration, and the percentage of the total amount of iron dialyzed (sum of iron in the apical, cell, and basolateral compartment) relative to the total iron in the 3-tier system (sum of iron in the digest, apical, cell, and basolateral compartment). Dialyzable iron was measured following both pepsin digestion alone and pepsin plus pancreatin-bile digestion. Values are means  $\pm$  SEM,  $n = 4$ . Means with no letters in common are significantly different,  $P < 0.05$ . For the lines without letters there is no differences in means.

**Figure 3** Iron uptake in 2 h from pepsin and pepsin plus pancreatin-bile digested rye bread with increasing amounts of lactate (0–200 mmol L<sup>-1</sup> initial concentration) in the 3-tier system. Values are means  $\pm$  SEM,  $n = 4$ . Means with no letters in common are significantly different,  $P < 0.05$ . For the line without letters there is no differences in means.

**Figure 4** The total iron uptake (sum of uptake and transcellular transport) relative to the amount of dialyzable iron in the 3-tier system following a 2 h incubation of pepsin and pepsin plus pancreatin-bile digested rye bread in the presence of increasing amounts of lactate from 0–200 mmol L<sup>-1</sup> initial concentration. Transcellular transport is not included for the pancreatic digests, but the total transport was only 53 pmol iron mg protein<sup>-1</sup> and not affected by the

1 lactate concentration. The correlations of iron absorption with dialyzable iron was  $R^2 = 0.41$   
2 and  $R^2 = 0.48$ , for the peptic and pancreatic digests respectively,  $P < 0.01$ . Values are means  $\pm$   
3 SEM,  $n = 4$ .









